AD_____

CONTRACT NUMBER: DAMD17-92-C-2006

TITLE: The Use of Diapausing Eggs from Killifish for Rapid, Sensitive Biological Screening of Toxicants in the Aquatic Environment.

PRINCIPAL INVESTIGATOR: Eugene Hull, Ph.D.

CONTRACTING ORGANIZATION: Orbis Scientific, Inc.
Pensacola, FL 32524

REPORT DATE: May 2, 1995

TYPE OF REPORT: Final - Phase II

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2, 1995	Final - Phase II (3 Dec 93 - 2 Apr 95)

4. TITLE AND SUBTITLE

The Use of Diapausing Eggs from Killifish for Rapid Sensitive Biological Screening of Toxicants in the Aquatic Environment

5. FUNDING NUMBERS

DAMD17-92-C-2006

6. AUTHOR(S)

Eugene Hull, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Orbis Scientific, Inc.
Pensacola, Florida 32524

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

A 108-tank recirculating system was constructed for the commercial breeding and maintenance of a large adult population of annual killifish, *Nothobranchius guentheri*. Embryos were harvested and successfully shipped to collaborating USABRDL for use in preliminary toxicant assessment studies. Several studies investigating the effects of temperature on Diapause III termination and hatching were conducted. Drops in temperature were found to terminate Diapause III and initiate the hatching process. Egg water loss induced by exposure to hypertonic NaCl solutions or dry storage in peat moss were able to inhibit temperature induced hatching. Results permitted the development of long term storage techniques for embryos to be used in portable toxicant assessment kits. Embryos that were preconditioned to enter Diapause II by adult exposure to short light cycle (10 hours light, 14 hours dark), removed from water and stored in peat moss at a temperature of 21°C, remained viable for 300 days. Results support the use of annual killifish eggs in a portable rapid toxicant assessment kit with a 90 day or greater shelf life.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Diapause - *Nothobranchius guentheri* - Toxicant assessment	54
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__✓__Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Eric Hall 5-2-95

PI - Signature Date

ACKNOWLEDGMENTS

I would like to thank the Research Methods Branch of the United States Army Biomedical Research and Development Lab for their support of this research.

I appreciate the care with which Mr. Tom Shedd, contracting officer's representative handled his end of the research and the time he took to review, constructively criticize and help us focus on the mission at hand. Through his efforts this project was concluded with positive results.

I want to thank Mr. Dan Signore, contracting specialist for his direction of our administrative staff.

TABLE OF CONTENTS

LIST OF TABLES

APPENDIX LIST

EXECUTIVE SUMMARY

During Phase I of this research, a 108 tank re-circulating aquaculture system was constructed for the maintenance of a large adult population (3,000 - 4,000) of *Nothobranchius guentheri* (*N. guentheri*). A series of range finding studies on the effects of water purity, hardness, alkalinity, filtration, salinity, and diet on growth and reproduction were accomplished in order to determine methodology for the economic mass culture of the annual killifish, *N. guentheri*. Additional range finding studies were conducted to determine the physical (light cycle and intensity) and chemical (water quality) conditions required for adult production of embryos that can be stored out of water while in a dormant stage of development (diapause) for a minimum of 90 days.

The constructed re-circulating aquaculture system that housed over 3,000 adult fish, provided an optimal breeding environment for these fish and over a half million eggs were harvested monthly. Range finding studies identified NaCl at concentrations of 3 - 4 parts per thousand (ppt) as an essential requirement for chorion strength and embryo survival. Adults grown in stock water containing less than 1 ppt NaCl produced embryos that demonstrated more than 90% mortality during development. No morphological defects were noted in the developing embryos, but the chorion (egg shell) surrounding the developing embryo was weak when compared to eggs from adults maintained at either 3 or 4 ppt NaCl. Embryo survival dramatically increased when collected from adults maintained at 3 or 4 ppt as NaCl. Embryos taken from adults maintained at 3 ppt NaCl were able to be maintained in our lab, out of water, on filter paper, for the target 90 day period required for the rapid toxicity test methods under development. The same eggs were able to be hatched within 30 minutes after being placed back in water.

During Phase II, a large population of *N. guentheri* continued to be cultured. Embryos were harvested and successfully shipped to collaborating USABRDL for use in preliminary toxicant assessment studies. Embryo handling methods were refined and various studies were conducted to determine why embryos did not continue to survive for the target shelf life of 90 days. During commercial shipment of embryos to collaborating United States Army Biomedical Research and Development Laboratory (USABRDL), egg mortality increased, chorion strength decreased and embryos prematurely hatched, despite being shipped in a semi-dry state. Further studies were performed to develop handling and packaging techniques that avoid the mortality associated with commercial shipment of these embryos. Lastly nutritional studies were conducted to improve feeding methodology developed during Phase I of this study.

Temperature drops associated with commercial shipment were found to be the causative agent for embryo mortality, hatching and most likely chorion weakening. The hatching process in killifish is initiated by the release of a hatching enzyme, in response to various environmental conditions (e.g. drop in oxygen tension). Embryos that were subjected to drops in temperature, similar to those experienced in commercial shipment were found to hatch or have a weakened chorion, perhaps due to the release of the hatching enzyme.

In order to further understand the hatching phenomenon and Diapause III termination, fully developed embryos, first subjected to either drying, induced water loss by exposure to hypertonic NaCl solution, diapause, water incubation or substrate manipulation were then exposed to a drop in temperature to determine if temperature induced hatching could be reduced or eliminated. In addition embryos, prior to full development were exposed to temperature drops to determine if mortality would increase due to chorion weakening.

Studies concluded that embryos rarely hatched, whether stored on paper or in water when held at a constant temperature. Exposure of embryos to a hypertonic NaCl solution prevented temperature induced hatching. Embryos that were not fully developed also failed to hatch or demonstrate chorion weakness when exposed to temperature drops. Utilizing these results, we developed a storage technique for the final rapid toxicity assessment kit. Embryos that were induced by photoperiod to enter Diapause II and stored in sterile soil to induce water loss, were able to be stored out of water for periods up to 300 days.

The problem of temperature drops experienced during commercial shipment of embryos was solved by the development of a simple package heater capable of heating the eggs during shipment. In addition, the new storage technique developed (storing eggs in sterile soil) reduced shipping associated mortality, increased embryo shelf life, and eliminated the complicated egg handling routines developed during Phase I of this contract.

In addition to refining handling techniques for embryos produced for toxicant assessment, a newly developed spirulina and yeast enriched brine shrimp was tested for enhanced growth in these fish. The enriched brine shrimp demonstrated better growth in these fish when compared to another commercially purchased non-enriched brine shrimp.

I. INTRODUCTION

In genetically predisposed species, diapause represents a state of development in which growth processes or conventional physiological functions are reversibly suppressed without injury to the organism. Diapause serves as a survival strategy which enables the species to cope with the harsh cyclical conditions of its normal habitat.

The manipulation of both diapause onset and termination would permit researchers to store embryos for extended periods of time without concomitant aging or change in cellular integrity. The present ability to terminate Diapause and hatch embryos within minutes makes the annual killifish an ideal candidate for the rapid biological assessment of toxicants. Through an understanding of embryonic diapause and the commercial production of the annual killifish, *Nothobranchius guentheri* (*N. guentheri*), several thousand embryos were harvested and made available to collaborating Research Methods Branch of the U.S. Army Biomedical Research and Developmental Laboratory (USABRDL) for rapid toxicant assessment studies. The present research outlines methods for manipulating Diapause in order to store embryos for extended periods of time. Methods of shipping embryos were developed which avoid drops in temperatures that were discovered to induce premature hatching.

N. guentheri (Teleostei: *Cyprinodontidae*) is an "annual killifish" found in the coastal regions of east Africa from Kruger Park to the Somali Republic and westward through the sub-Saharan basin to Gambia (Turner, 1964; Klee, 1965; Haas, 1976a,b). They are found in ponds having mud substrates and subjected to total seasonal drying (Jubb, 1969). *N. guentheri* (Pfeiffer) is found in the coastal lowlands of Tanzania and Kenya (Bailey, 1972). The annual fish are unique in that they are common only in habitats which are subject to complete seasonal drying and erratic climatic conditions, while being rare in permanent bodies of water (Myers, 1952; Bailey, 1972; Wourms, 1972a,b,c). As their habitats dry, all hatched fish die and the species survival is totally dependent on the adult's substrate deposition of drought resistant eggs that are capable of entering diapause (Peters, 1963; Wourms, 1964). These eggs remain in a state of diapause until hatching is triggered by the onset of rainfall. Other reproductive modifications include early sexual maturity, daily egg production, and a moderately high primary reproductive effort (Simpson, 1979).

In most oviparous fish, a continuous mode of embryonic development follows fertilization (Oppenheimer, 1937, 1947; Trinkaus, 1951,1963 & Ballard, 1973). Manipulation of factors to slow development (e.g. oxygen, salinity, temperature) tends to increase morbidity and malformity (reviewed in Inglima, 1980). In contrast, the annual killifish may exhibit an embryonic diapause of variable duration at three specific stages of development (Peters, 1963; Wourms, 1964, 1972a,b,c) without a corresponding increase in morbidity and malformation. The incidence of diapause has been shown to be controlled by such biophysical factors as light-dark cycle, season of collection (Markofsky et al., 1979), temperature (Markofsky and Matias, 1977, 1978), oxygen (Peters, 1963) and intrinsic factors such as adult diffusable substances (Inglima et al., 1981). The stages of

development described by Wourms for *Austrofundulus* have been noted to be similar for *Nothobranchius* and are outlined below for those stages where diapause may occur.

Diapause I (DI) occurs at stage 20 (Dispersed Phase one) after completion of epiboly. At this time there exists an amoeboid cell population which remains randomly distributed within the space between the two concentric layers, the periblast and enveloping layer. Diapause II (DII) occurs at stage 32-33 when there is a long somite embryo (35-42 somites) just prior to the initiation of circulation. If DII occurs at stage 32, the heart appears as a simple non-contracting tube. If DII occurs at stage 33, the heart may exhibit erratic contractions. When a few colorless blood cells are observed circulating, and the heart beats at a steady rate, DII can be considered terminated. Diapause III (DIII) occurs at stage 43 during full embryonic development just prior to hatching (Wourms, 1972a). In *N. guentheri*, D I is not observed unless induced by reduced oxygen tension (Peters, 1963; Inglima et al., 1981), hypothermia (Markofsky & Matias, 1978) and adult diffusable substances (Inglima, 1980). D II is controlled by the light cycle and the intensity and duration of exposure (Dose of Light) (Hull 1986). D I and D II, by their ability to be expressed only by induction by certain environmental stimuli, are called facultative (Wourms, 1964, 1966). D III, on the other hand is obligate and almost invariably occurs. A few eggs bypass diapause and Wourms has called these "escape eggs". Historically, Diapause has been noted to be highly variable even when maintained under laboratory constant conditions. Work by Hull (1986) demonstrated that several environmental factors when applied together, will modify the onset and duration of Diapause in a predictable fashion.

Methods developed to commercially produce these fish as well as control diapause in a predictable/reproducible fashion, not only permit the development of a portable and rapid toxicant assessment kit, but allow for sensitive biochemical studies aimed at isolating "Diapause Factor" which is currently believed to be passed from the adult female to the ova in response to various environmental stimuli.

II. RATIONALE

Previously, in Phase 1 of this research we discovered several factors that improve egg viability, and were able to store eggs for the target 90 day period. Methodology was also developed that permitted collaborating labs to rapidly hatch fish. This called for embryos to be water incubated for 35 days, followed by storage on moist filter paper in petri dishes for an additional 10 - 15 days. The resulting egg hatched rapidly, within 30 minutes, when submerged in water. The same methodology created a new problem in that the egg, once removed from water, was more sensitive to shipping and handling and subject to premature hatching and high mortality. Phase II research efforts explored the effects of temperature drops on hatching in eggs incubated under differing conditions. Results were used to develop methods which prevent mortality tied to shipping induced temperature fluctuation. In addition, new storage techniques were tested that simplify handling of these eggs, while extending their shelf life. Methods were tested by actual shipment of eggs to collaborating USABRDL.

III. MATERIALS AND METHODS

Fish

Laboratory populations of *N. guentheri* were derived from an original strain maintained at the Orentreich Foundation for Science (N.Y., N.Y.) in 1983. This population was crossed with several other populations maintained by members of the American Killifish Association during 1990-1992. Prior to the cross, eggs were harvested from these fish and inspected for diapause to insure their acting similarly in response to light. Fish were routinely selected for healthy characteristics, such as body shape, color, maximum growth, longevity, egg production, and rapid rate of growth. Information on the original population and methods of breeding, egg collection, hatching and raising fry has been reported in the following articles: Markofsky and Perlmutter, 1972, 1973; Inglima, 1980; Hull, 1986, Report # AD-A289056).

Facility & Equipment

A temperature controlled 5,000 square foot insulated building located at 109 SW Caroline Street, Milton Florida was utilized as a laboratory. Adult fish were maintained in experimental tanks consisting of 5 gallon static aquaria. The general adult population was maintained in one hundred 30 gallon tanks linked together to create a recirculating system. Fish were hatched and raised in PVC pools of various capacity covered by a green house. Fish were transported to the lab as needed when mature. A complete description of the tanks, facility design, and breeding methodology was given in the Phase I report (Report # AD-A289056).

Water Treatment

Water was pumped from a 105 feet deep well. All water lines were PVC plastic. Well water was passed through a commercially installed Culligan water purification system that permitted water to be taken after various stages of filtration. All well water was prefiltered down to 1 micron. Water passed through carbon and was used at this point or further purified by a water softener and thin film composite reverse osmosis. Water used for embryo research and holding of the adult population was a 50:50 mix of carbon filtered well water and carbon filtered well water further treated by reverse osmosis. This water was stored and aerated for a minimum of 2 days in 800 gallon high density polyethylene tanks. Water was conditioned by the addition of $CaC0_3$ and NaCl. Other trace chemicals used are fully described in Report # AD-A289056.

Water Conditions

The general adult and embryo stock water conditions are listed below:

pH	6.8-7.1
Alkalinity	17-34 ppm as $CaC0_3$
Hardness	17-34 ppm as $CaC0_3$
Salinity	3 parts per thousand as NaCl

Additional information on water treatment and storage can be found in the report covering Phase I of this research.

Food

Adult fish were fed a combination of frozen or live blood worms, adult brine shrimp, standard aquarium flake food, freeze dried blood worms, and Daphnia. Food preparation and regimen can be found in detail in the report covering Phase I of this research. In the appendix of this report, we discuss a new nutritionally supplemented food source which benefited the growth of these fish. This new supplemented brine shrimp replaced the vitamin fortified paste, reported in Phase I, which was laborious to produce.

Lighting

Light controlled chambers measuring 1.2m long x 0.8m high and 0.8m wide were constructed out of wood, with overhead cool white fluorescent lights (General Electric, 2 pin, 48 inch). The light intensity was measured daily at the surface of the water during the first two weeks and twice per week thereafter using a Lux Meter (Pet Warehouse). The intensity of light was set by changing the distance between the aquarium and the fluorescent fixtures. Each tank was surrounded by brown cardboard so only overhead illumination was responsible for light reaching the tank. The light cycle in each chamber was controlled by automatic electric timers to produce long day photoperiods consisting of 14 hours of light and 10 hours of darkness (L:D, 14:10) and short day photoperiods

consisting of 10 hours of light and 14 hours of darkness (L:D, 10:14). Water temperature was monitored daily and found to be uniform throughout the chambers. Temperature varied between 24.0°C and 26.0°C.

Diapause Control

Methods developed by Hull (1986) were used to manipulate adult fish to produce embryos that either enter or bypass Diapause. Adults that produce embryos that enter D II were exposed for 100 or more days to short day conditions (L:D, 10:14). Adults that produce embryos that bypass D II were exposed to long day conditions (L:D, 14:10). Intensity of light was 150 ft-candles (1500 Lux).

General Handling of Fish and Embryos

Unless otherwise noted, each experimental tank bottom was covered by a 2-3 cm layer of fine white marine sand and contained 16 liters of stock water adjusted to 3 grams per liter with NaCl. All experimental tanks were 5 gallon glass tanks (40cm x 21cm x 26cm) and contained one rectangular filter at the base, as recommended by Wourms (1966). Each filter contained two layers of aquarium floss with 1-2 cms of gravel (Estes #3) and 1.6 grams boiled peat moss (Fluval) sandwiched between them. To begin each experiment, 80 day old fish were selected from the general stock and randomly assigned to each tank. In every experimental design, the adult number and sex ratio among aquaria were kept constant. Each spawning group consisted of 3 capable females and 2 capable males. Each experimental group consisted of 4 five gallon aquaria labeled A - D. The composition was maintained by replacing fish that died with a fish of the same age that had been exposed identically. One third of the water was replaced with fresh stock water weekly. Eggs were harvested by stirring up the sand and swirling a fine mesh net in the tank in a figure 8 motion. A five day spawning period was selected to allow for a sufficient accumulation of embryos without allowing the embryo population to differ widely in age. Discarding the eggs in the aquaria 5 days prior to an egg collection insured all eggs to be 1-5 days old at the start of our observations.

Eggs were collected and immediately placed in glass trays (24"x12"x4") containing fresh stock water which was changed daily to prevent bacterial build up. Eggs were maintained in the dark at room temperature (24-26°C) until they reached the desired stage of development.

Prior to any experiment, eggs were inspected using a stereomicroscope (40X). The position of the developing embryo, clarity of the chorion and characteristic double membrane (a unique characteristic of the annual killifish (Wourms, 1966) were used to determine viability and embryo condition. When water incubated, viable embryos were selected and placed in glass bowls (14cm diameter and 5cm high). If eggs were to be dried, they were placed on filter paper (Whatman #1) and sealed with Parafilm in Falcon brand petri-dishes (90 x 15 mm). For peat studies, embryos were placed in Canadian Peat moss that was first boiled several times to remove impurities and reduce ammonia. The

peat moss was sealed in Falcon brand petri dishes (90 x 15 mm) using Parafilm. The peat moss and filter paper were dried by blotting until slightly moist to the touch.

Embryos used for various temperature experiments were maintained at 21.0°C in a water bath (Aquanetics) or at 25.0°C in a Precision Model 818 low temperature incubator. Temperature range varied $\pm$ 0.5 °C.

Experimental eggs were inspected on specific days set forth in each experimental design. Visual inspections took place at room temperature under a large 3X viewing glass or stereomicroscope. Therefore, embryos that were incubated at 21.0°C experienced a slight rise in temperature during the inspection process. Eggs at 25.0°C experienced a negligible change in temperature since room temperature was approximately 25.0°C. Embryos were considered hatched if they were observed free swimming or completely free of the egg chorion. For a more detailed description of handling, see Phase I report.

Statistical Analysis

Data generated from hatching studies were analyzed for each day utilizing both an exact nonparametric permutation test and an asymptotic X^2 test with the software StatXact-Turbo. A repeated measures design was utilized to analyze the growth study. Statistical results are given in Appendix IV.

IV. EXPERIMENTAL DESIGN

A. The effect of drops in temperature on hatching and viability in water incubated eggs. Adults were exposed to non diapause inducing long day conditions (L:D, 14:10) for 100 days.

This study was performed to determine the effect of shipping related drops in temperature on hatching and viability. The understanding of factors that trigger the hatching process and the termination of D III, will permit the development of methods that reduce shipping associated mortality and premature hatching.

Five eighty-day-old fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to long day conditions (L:D, 14:10) for 100 days at an intensity of 150 ft-cs. Adults exposed to these conditions were previously noted to produce embryos that bypass D I & D II. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank and placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop over a 35 day period at room temperature to stage 43. The embryos were visually inspected under a stereomicroscope at 40X magnification to insure normal development and membrane morphology. Sixty eggs were selected from each of the four groups and placed 30 per glass bowl in 200 milliliters of freshly prepared stock water. One set of 4

bowls was placed in an incubator at a temperature of 25.0°C and labeled A-D control. The remaining set of 4 bowls was placed in a water bath at 21.0°C, representing a 4.0°C drop in temperature. Temperature was checked after 3 hours to insure the target temperature (21°C) was reached. Variation in both incubators was $\pm$ 0.5°C. A 50% water change was conducted every fourth day with identical water at the same temperature. Eggs were inspected visually on days 4, 8, 12 & 20 and viability and hatching noted (Table 1).

B. The effect of drops in temperature on hatching and viability in water incubated eggs that were dried for 10 days prior to being returned to water incubation. Adults were preconditioned to produce embryos that bypass D II by adult exposure to long day conditions (L:D, 14:10) for 100 days.

This study was performed to determine how eggs that experience a brief 10 day drying period react to shipping related drops in temperature. The understanding of factors that trigger the hatching process and the termination of D III will permit the development of methods that reduce shipping associated mortality and premature hatching.

Five eighty-day-old adult fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to long day conditions (L:D, 14:10) for 100 days at an intensity of 150 ft-cs. Adults exposed to these conditions were previously noted to produce embryos that bypass Diapause I & II. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank and placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop over a 35 day period at room temperature to stage 43. The embryos were visually inspected under a stereomicroscope at 40X magnification to insure normal development and membrane morphology. Seventy eggs were selected from each of the four groups and dried by placement on moistened Whatman filter paper, 35 per filter, and sealed in petri-dishes using parafilm. Two sets of dishes labeled A-D control and A-D experimental were placed in an incubator at 25.0°C. After 10 days the experimental dishes were removed, 30 viable embryos were selected from each dish, placed in bowls containing freshly prepared stock solution at 25°C, and transferred to a 21.0°C water bath. The water temperature was monitored after 3 hours to insure the embryo incubating solution reached 21°C. The controls were treated similarly but were left at 25.0°C. Eggs were inspected visually on days 4,8,12 & 20 and viability and hatching noted (Table 2).

C. The effect of drops in temperature on hatching and viability in dried eggs that are stored on moist filter paper. Adults were preconditioned to produce embryos that bypass DII by adult exposure to long day conditions (L:D, 14:10) for 120 days.

This study was performed to determine the effect of shipping related drops in temperature on hatching and viability in dried eggs. Similar conditions are experienced by the eggs shipped to collaborating laboratories. The understanding of factors that trigger the

hatching process and the termination of D III, will permit the development of methods that reduce shipping associated mortality and premature hatching.

Five eighty-day-old adult fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to long day conditions (L:D, 14:10) for 120 days at an intensity of 150 ft-cs. Adults exposed to these conditions were previously noted to produce embryos that bypass D I & D II. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank and placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop over a 35 day period at room temperature to stage 43. The embryos were visually inspected under a stereomicroscope at 40X magnification to insure normal development and membrane morphology. Seventy eggs were selected from each of the four groups and placed on moistened Whatman filter paper, 35 per filter, and sealed in petri-dishes. Two sets of dishes labeled A-D control and A-D experimental were placed in an incubator at 25.0°C. After 10 days, the experimental dishes were removed, 30 viable embryos (Age = 45 days) were selected from each dish, placed on new filter paper, and transferred to 21.0°C. The controls were treated similarly but were left at 25.0°C. Eggs were inspected visually on days 4,8,12 & 20 and hatching noted (Table 3). Eggs that had thin chorions and crushed when gently rolled by hand were considered hatched. The assumption was made that the hatching process (hatching enzyme released) occurred , but fish could not completely shed the chorion due to the lack of surrounding water.

D. The effect of drops in temperature on hatching and viability in water incubated eggs that entered D II for 20 days. Eggs were dried for 10 days prior to being returned to water incubation. Adults were programmed to produce embryos that enter D II by exposure to short day conditions (L:D, 10:14) for 100 days.

This study was performed to determine the effect of drops in temperature on hatching in eggs that enter D II for 20 days prior to being subjected to a 10 day drying period. Similar conditions are experienced by the eggs shipped to collaborating laboratories. The understanding of factors that trigger the hatching process and the termination of D III, will permit the development of methods that reduce shipping associated mortality and premature hatching.

Five eighty day old adult fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to short day conditions (L:D, 10:14) for 100 days at an intensity of 150 ft-cs. Adults exposed to these conditions were previously noted to produce embryos that enter D II. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank and placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop

over a 55 day period at room temperature to stages 43 (prehatching stage). These embryos which normally develop to the prehatching stage in 35 days entered a dormant stage of development (D II) for 20 days bringing their total developmental time to 55 days. The embryos were visually inspected under a stereomicroscope at 40X magnification to insure normal development, diapause onset and membrane morphology. Seventy eggs were selected from each of the four groups and placed on moistened filter paper, 35 per filter, and sealed in petri-dishes. Two sets of dishes labeled A-D control and A-D experimental were placed in an incubator at 25.0°C. After 10 days the experimental dishes were removed, 30 viable embryos (Age = 65 days) were selected from each dish, placed in bowls containing freshly prepared stock solution, and transferred to 21.0°C. Temperature was checked after 3 hours to insure the experimental conditions (21°C) were reached. The controls were treated similarly but were left at 25.0°C. Eggs were inspected visually on days 4,8,12 & 20 and viability and hatching noted (Table 4).

E. The effect of drops in temperature on hatching and viability in water incubated eggs, that are developed to stage 38-40, prior to the pre-hatching stage (D III). Adults were exposed to long day conditions (L:D, 14:10) for 120 days.

This study was performed to determine the effect of shipping related drops in temperature on hatching and viability in eggs that are not fully developed. Mortality experienced during transport, which may be due to temperature fluctuation, may be avoided if embryos are shipped to collaborating labs prior to the prehatching stage (D III).

Five eighty day old adult fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to long day conditions (L:D, 14:10) for 120 days at an intensity of 150 ft-cs. Adults exposed to these conditions were previously noted to produce embryos that bypass Diapause I & II. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank, placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop over a 20-25 day period at room temperature to stages 38-40. The embryos were visually inspected under a stereomicroscope at 80X magnification to insure adequate/normal development and membrane morphology. Sixty eggs between stages 38-40 were selected from each of the four groups and placed, 30 per glass bowl, in 200 milliliters of freshly prepared stock water. One set of 4 bowls was placed in an incubator at a temperature of 25.0°C and labeled A-D control. The remaining set of 4 bowls were placed in a water bath at 21.0°C, representing a 4.0°C drop in temperature. Temperature was checked in 3 hours to insure experimental conditions (21°C) were reached. Variation in both incubators was $\pm$0.5°C. Eggs were inspected visually on days 4,8,12 & 20 and viability and hatching noted (Table 5).

F. The effect of reducing egg water content on hatching and viability in embryos exposed to a 4.0°C drop in temperature known to induce hatching. Adult fish were exposed to long day conditions (L:D, 14:10) for 130 days.

This study was performed to determine the effect of drops in temperature on hatching in eggs experiencing water loss induced by exposure to hypertonic NaCl solutions. Many observations on embryos stored in peat moss indicate embryos are less likely to hatch when shipped during cool temperatures. The removal of water via exposure to a hypertonic solution of NaCl was meant to mimic the water loss that takes place when eggs are stored in peat moss.

Five eighty day old adult fish of a similar size were randomly placed in each of 4 experimental aquaria at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to long day conditions (L:D, 14:10) for 130 days at an intensity of 150 ft-cs. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank, placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop at room temperature to stages 25-32. The embryos were visually inspected under a stereomicroscope at 80X magnification to insure adequate/normal development and membrane morphology. Sixty eggs between stages 25-32 were selected from each of the four groups and placed 30 per glass bowl in 200 milliliters of freshly prepared stock water. One set of 4 bowls was placed in an incubator at a temperature of 25.0°C and labeled A-D control. The remaining set of 4 bowls had their incubating solution changed with 10 ppt NaCl stock solution. These bowls were labeled A-D experimental and placed in the incubator at 25.0°C. When the experimental embryos demonstrated shrinkage of the primary membrane from the chorion and reached the prehatching stage, they were removed and placed at 21.0°C. Temperature was checked after 3 hours to insure experimental conditions (21°C) were reached. Control eggs were left at 25.0°C. Eggs were inspected visually on days 4,8,12 & 20 and viability and hatching noted (Table 6).

G. The effect of short day adult exposure, (L:D, 10:14) on long term storage of embryos dried in peat moss and stored at 21°C. Adult fish were exposed to short day photoperiods for 130 days.

To maintain large quantities of annual killifish is costly and only viable when a large research effort is taking place. For economic purposes as well as for convenience, it would be beneficial to store embryos for extremely long periods. The cost of storage is negligible, and avoids the chronic attention a continuous breeding facility necessitates. Three factors known to govern Diapause onset and or duration were studied together to determine if embryos could be stored for 300 days. Diapause onset was induced by 130 day adult exposure to short day photoperiods which were previously observed to induce embryos to enter D II. These embryos were stored in dry peat which was moistened by the addition of a small amount of water, which leads to evaporative water loss, known to

lengthen the duration of D II. Lastly, the embryos will be stored at 21.0°C, a temperature known to either lengthen or induce D II.

Five eighty day old adult fish of a similar size were randomly placed in each of 4 experimental aquaria labeled A-D, at a 3:2 female to male ratio. Aquaria and fish were set in light boxes and exposed to short day conditions (L:D, 10:14) for 130 days at an intensity of 150 ft-cs. Tanks were maintained at room temperature which varied between 24.0° and 26.0°C. Eggs between 1 and 5 days of age were collected from each tank and placed in labeled (A-D) glass trays (24"x12"x4"), and water incubated in stock 3 ppt NaCl solution. The incubation solution was changed daily while the embryos were permitted to develop at room temperature to stages 28-31 just prior to the onset of D II.. Embryos previously collected from these adults were observed to enter D II. The embryos were visually inspected under a stereomicroscope at 80X magnification to insure normal development and membrane morphology. Thirty five eggs were selected from each of the four groups and placed in large peat filled petri dishes and sealed with parafilm. The dishes were labeled A-D and placed at 21.0°C. After five days, 30 viable embryos were selected from each dish and placed in new identically labeled dishes, containing sterile boiled peat moss. Embryos were placed back at 21.0°C. Eggs were inspected every 90 days and on day 300. Data was recorded as number of eggs less than or equal to D II or greater than D II, and number viable. Results are reported in Table 7.

V. EXPERIMENTAL RESULTS

The effect of lowering the incubation temperature of the embryo on hatching and viability.

A. The effect of drops in temperature on hatching and viability in water incubated eggs. Adults were exposed to non diapause inducing long day conditions (L:D, 14:10) for 100 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Very few eggs (3.4%) hatched in the control group incubated at a constant temperature of 25.0°C. There was a slightly higher incidence of hatching (13.8%) in eggs exposed to a 4.0°C drop in temperature. Survival in control eggs was slightly higher than eggs subjected to a drop in temperature, reported as 95.8% and 90.8% respectively. It appears that the non-viable embryos did not suffer from abnormal deaths, but failed to hatch completely from the egg shell. The chorions of the non-viable eggs were always paper thin, indicating enzymatic digestion from within, but were not picked up as hatched since criteria for hatching was complete escape from the chorion. Once a fish hatched it was discarded but carried forward in the data as viable and hatched. Data is presented below in Table 1.

Table 1. The effect of embryo exposure to a 4.0°C drop in temperature on hatching and viability. Eggs were water incubated and permitted to develop to Diapause III. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were randomly split into two groups of 30 and placed at either 25.0°C or 21.0°C. Data are reported as number hatched/ number viable on days 4,8,12, & 20. Total hatched/viable, percent hatched and survival are given for day 20. The days on which significant differences appear are noted in bold type.

	CONTROL 25°C # Hatched/#Viable				EXPERIMENTAL 21°C # Hatched/#Viable			
DAY	A	B	C	D	A	B	C	D
0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
4	**0/30**	**0/30**	**0/30**	**0/30**	**2/29**	**1/30**	**0/30**	**0/30**
8	**0/30**	**0/30**	**1/30**	**1/29**	**3/27**	**1/30**	**2/29**	**2/29**
12	**0/30**	**0/29**	**2/29**	**1/29**	**5/27**	**3/29**	**2/28**	**3/28**
20	**0/29**	**0/28**	**3/29**	**1/29**	**5/26**	**3/28**	**2/28**	**5/27**
Total # Hatched/Viable		4/115				15/109		
Percent Hatched		3.5%				13.8%		
Survival (%)		95.8%				90.8%		

B. The effect of drops in temperature on hatching and viability in water incubated eggs that were dried for 10 days prior to being returned to water incubation. Adults were pre-conditioned to produce embryos that bypass D II by adult exposure to long day conditions (L:D, 14:10) for 100 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Embryos that were dried for 10 days and exposed to a drop in temperature demonstrated lower survival (82.5%) and a higher hatch rate (41.4%) when compared to controls. Control survival and hatch rate was 88.3% and 7.5% respectively (Table 2). As in Experiment A, mortality was not due to abnormal circumstances, but was most likely embryos that failed to completely break out of their egg chorion. Data is presented below in Table 2.

Table 2. The effect of embryo exposure to a 4.0°C drop in temperature on hatching and viability. Eggs were water incubated until fully developed, dried for 10 days, then returned to water incubation. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were randomly split into two groups of 30 and placed at either 25.0°C or 21.0°C. Data are reported as number hatched/ number viable on days 4,8,12, & 20. Total hatched/viable, percent hatched and survival are given for day 20. The days on which significant differences appear are noted in bold type.

	CONTROL 25°C # Hatched/#Viable				EXPERIMENTAL 21°C #Hatched/#Viable			
DAY	A	B	C	D	A	B	C	D
0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
4	**1/29**	**0/30**	**1/29**	**0/30**	**2/29**	**4/30**	**1/28**	**6/28**
8	**1/28**	**0/29**	**2/28**	**1/28**	**5/28**	**6/28**	**4/27**	**6/27**
12	**2/28**	**1/27**	**2/27**	**1/27**	**13/26**	**7/28**	**8/26**	**6/27**
20	**2/27**	**1/27**	**4/26**	**1/26**	**14/23**	**9/25**	**11/25**	**7/26**
Total# Hatch/Viable	8/106				41/99			
Percent Hatched	7.5%				41.4%			
Survival	88.3%				82.5%			

C. The effect of drops in temperature on hatching and viability in dried eggs that are stored on moist filter paper. Adults were preconditioned to produce embryos that bypass D II by adult exposure to long day conditions (L:D, 14:10) for 120 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Eggs that were dried while exposed to a drop in temperature demonstrated a higher hatch rate (15.0%) than controls (3.3%) (Table 3). Survival was assumed perfect, since eggs that were crushed when lightly touched were recorded as hatched. Since the eggs are stored out of water, an embryo could not burst out of the chorion (criteria used for hatching). The enzyme would digest the chorion if hatching was initiated, and the egg would crush if gently touched.

Table 3. The effect of embryo exposure to a 4.0°C drop in temperature on hatching. Eggs were water incubated until fully developed and then transferred to moist filter paper. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were randomly split into two groups of 30 and placed at either 25.0°C or 21.0°C. Data are reported as number hatched on days 4,8,12, & 20. Total number and % hatched are given for day 20. The days on which significant differences appear are noted in bold type.

	CONTROL 25°C # Hatched				EXPERIMENTAL 21°C #Hatched			
DAY	A	B	C	D	A	B	C	D
0	30	30	30	30	30	30	30	30
4	**0**	**1**	**0**	**0**	**1**	**2**	**2**	**1**
8	**0**	**1**	**0**	**0**	**1**	**3**	**5**	**1**
12	**1**	**1**	**0**	**0**	**1**	**5**	**5**	**2**
20	**2**	**1**	**0**	**1**	**2**	**5**	**8**	**3**
Total # Viable		120				120		
Total # Hatch*		4				18		
Percent Hatch		3.3%				15.0%		

* Number Hatched in eggs stored out of water were those which crushed to gentle touch. It was assumed that the hatching enzyme was triggered, but hatching prevented due to the lack of water.

D. The effect of drops in temperature on hatching and viability in water incubated eggs that were programmed to enter D II for 20 days. Eggs were dried for 10 days prior to being returned to water incubation. Adults were programmed to produce embryos that enter D II by exposure to short day conditions (L:D, 10:14) for 100 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Embryos that were observed to enter D II for 20 days were selected for study. Embryos that were exposed to a 4.0°C drop in temperature demonstrated a higher hatch rate (46.7%) than controls (8.3%) maintained at 25.0°C. Survival was lower in embryos exposed to a drop in temperature (87.5%) when compared to controls (90.8%).

Table 4. The effect of embryo exposure to a 4.0°C drop in temperature on hatching and viability. Eggs that entered D II for 20 days were water incubated until they were fully developed, transferred to moist filter paper for 10 days and finally returned to aqueous incubation. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were split into two groups of 30 and randomly placed at either 25.0°C or 21.0°C. Data are reported as number hatched/ number viable on days 4,8,12, & 20. Total hatched/viable, percent hatched and survival are given for day 20. The days on which significant differences appear are noted in bold type.

DAY	CONTROL 25°C # Hatched/#Viable				EXPERIMENTAL 21°C #Hatched/#Viable			
	A	B	C	D	A	B	C	D
0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
4	**0/29**	**2/30**	**2/28**	**0/29**	**4/28**	**6/30**	**2/30**	**3/29**
8	**2/28**	**2/29**	**2/28**	**1/29**	**11/27**	**7/29**	**3/29**	**5/28**
12	**2/28**	**3/28**	**2/27**	**2/28**	**16/27**	**12/28**	**8/27**	**6/28**
20	**2/27**	**3/28**	**2/26**	**2/28**	**17/27**	**15/26**	**11/26**	**6/26**
Total # Hatch/Viable	9/109				49/105			
Percent Hatch	8.3%				46.7%			
Survival	90.8%				87.5%			

E. The effect of drops in temperature on hatching and viability in water incubated eggs that are developed to stage 38-40, prior to the pre-hatching stage. Adults were exposed to long day conditions (L:D, 14:10) for 120 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Embryos, not fully developed, exposed to a drop in temperature of 4.0°C, led to a hatch rate of less than 5%, similar to the control hatch rate of 3.4%. Survival was slightly higher in control eggs (97.5%) compared to experimental (93.3%) (Table 5). Eggs did not begin hatching until day 12, in comparison to day 4 in previous experiments. No significant differences existed between groups.

Table 5. The effect of embryo exposure to a 4.0° C drop in temperature on hatching and viability in eggs that are not fully developed (staged between 38-40). Eggs were water incubated throughout development. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were randomly split into two groups of 30 and placed at either 25.0°C or 21.0°C. Data are reported as number hatched/number viable on days 4,8,12, & 20. Total hatched/viable, percent hatched and survival are given for day 20.

	CONTROL 25°C				EXPERIMENTAL 21°C			
DAY	# Hatched/#Viable				#Hatched/#Viable			
	A	B	C	D	A	B	C	D
0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
4	0/30	0/30	0/30	0/30	0/30	0/30	0/28	0/30
8	0/30	0/30	0/30	0/30	0/29	0/30	0/28	0/30
12	0/29	1/30	0/28	2/30	0/28	0/30	0/28	0/30
20	0/29	1/30	1/28	2/30	3/28	2/29	0/27	0/28
Total # Hatch/Viable		4/117				5/112		
Percent Hatch		3.4%				4.5%		
Survival		97.5%				93.3%		

F. The effect of reducing egg water content on hatching and viability in embryos exposed to a 4.0°C drop in temperature known to induce hatching. Adult fish were exposed to long day conditions (L:D, 14:10) for 130 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. The primary membrane was observed to be shrunk away from the chorion in eggs exposed to a hypertonic NaCl solution. By reducing the water content of the egg by exposure to a hypertonic NaCl solution, the temperature induced hatching process was inhibited, with no eggs hatching in response to temperature change. Survival for treated eggs was 87.5% compared to 95.0% for controls. Few eggs (3.5%) hatched in controls supporting previous findings in Experiment A. By comparing the results of this study to previous studies (A-D), it becomes obvious that exposure to hypertonic solutions prevents the temperature induced hatching response.

Table 6. The effect of embryo exposure to a 4.0°C drop in temperature on hatching and viability. Experimental eggs were exposed to a hypertonic NaCl solution and water incubated throughout development. Sixty eggs were sampled from each of four aquaria. Eggs from each aquaria were randomly split into two groups of 30 and placed at either 25.0°C or 21.0°C. . Data are reported as number hatched/ number viable on days 4,8,12, & 20. Total number hatched/viable, percent hatched and survival are given for day 20. The days on which significant differences appear are noted in bold type.

	CONTROL 3 g/L 25°C				EXPERIMENTAL 10 g/L 21°C			
DAY	# Hatched/#Viable				Hatched/#Viable			
	A	B	C	D	A	B	C	D
0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
4	0/30	0/30	0/30	0/29	0/30	0/30	0/30	0/30
8	0/30	0/29	0/29	1/29	0/30	0/30	0/27	0/30
12	**1/29**	**1/28**	**0/29**	**1/29**	**0/29**	**0/28**	**0/26**	**0/28**
20	**1/29**	**1/28**	**0/29**	**2/28**	**0/28**	**0/26**	**0/26**	**0/25**
Total # Hatch/Viable	4/114				0/105			
Percent Hatch	3.5%				0%			
Survival	95.0%				87.5%			

G. The effect of short day adult exposure, (L:D, 10:14) on long term storage of embryos dried in peat moss and stored at 21°C. Adult fish were exposed to short day photoperiods for 130 days.

When visually inspected under the microscope, embryos were free of abnormalities and membranes exhibited normal morphology. Over 89% of the embryos survived for 180 days or longer. Over 50% of the embryos bypassed DII by day 270. D II may have been terminated early due to the temperature fluctuations (21-26°C) experienced while visually inspected at room temperature on days 90, 180, 270 and 300. Point estimates and 95% confidence intervals at each inspection period are given in Appendix IV.

Table 7. Survival and duration of D II in embryos from short day exposed adults. Eggs were stored in peat moss at a diapause inducing temperature of 21.0°C. Thirty eggs were sampled from each of four aquaria. Data are reported as number and total number of embryos developed less than or equal to DII (stage 32), greater than DII, and number viable. Eggs were inspected on days 90, 180, 270, and 300.

Day	Tank	#≤DII	# > DII	#Viable
	A	26	2	28
90	B	30	0	30
	C	29	0	29
	D	27	1	28
Total(%)		***112(97)***	***3(3)***	***115(96)***
	A	18	8	26
180	B	22	6	28
	C	26	2	28
	D	19	6	25
Total(%)		***85(79)***	***22(21)***	***107(89)***
	A	5	12	17
270	B	8	16	24
	C	13	7	20
	D	10	11	21
Total(%)		***36(44)***	***46(56)***	***82(68)***
	A	2	11	13
300	B	1	20	21
	C	5	10	15
	D	7	9	16
Total(%)		***15(23)***	***50(77)***	***65(54)***

DISCUSSION

The various studies conducted clearly demonstrate drops in temperature to be responsible for triggering the hatching process and terminating D III. Results explain the high mortality and weak chorions reported by collaborating USABRDL for those embryos shipped in the winter months. Three methods born from this research were developed to provide a solution to the hatching witnessed during winter shipment of embryos. The first method utilizes the developed "package heater" that is driven by a battery to generate small quantities of heat. The package heater and eggs are placed in a 2 inch thick foam container which insulates the eggs from ambient conditions, while reducing heat loss from the battery operated package heater. Keeping the embryos from experiencing a drop in temperature will reduce mortality associated with shipping in colder months. The design of the battery operated package heater is given in Appendix 1. The second method requires embryos to be shipped at a stage of development where hatching does not occur (prior to stage 42-43). In Experiment E, no stage 38-40 embryos hatched when exposed to a temperature drop for 8 days. This method is not advantageous, since test facilities would have to maintain embryos in their labs prior to use. The last method developed employs a combination of evaporative water loss and storage in peat moss. Embryos stored in peat moss have been observed routinely to ship with low mortality in cold weather and avoid the premature hatching witnessed in eggs presently shipped on moist filter paper.

Collaborating researchers had difficulty in maintaining viability of embryos stored on filter paper, but reported favorable results when embryos were stored and shipped in peat moss. In addition, embryos stored in peat moss demonstrated extremely high survival rates during investigations which required prolonged handling during visual inspection (Experimental Design G). Embryos demonstrated 96% survival after 90 days and 87.5% after 180 days. Fifty four percent of the embryos survived a 300 day incubation period in peat moss. These results, when compared to previous findings during Phase I, indicate that a peat substrate is superior to a paper substrate in embryo survival and shelf life. In addition, no handling or maintenance is required for eggs stored in peat, whereas eggs stored on filter paper require weekly observation and re-plating. The effects of hypertonic solutions on hatching (Experimental Design F) identifies why embryos stored in dry peat fail to hatch when exposed to cool temperatures. Eggs in dry peat tend to lose water to the surrounding peat which acts as a sponge. Since it is difficult to observe embryos in peat, eggs were subject to hypertonic solutions that mimic the effects of peat on eggs. The egg membranes clearly shrunk due to water loss and failed to hatch when exposed to temperatures previously observed to hatch eggs. Embryos stored in peat moss permits researchers to maintain dormant embryos for extended periods of time, without premature hatching caused by small changes in temperature. This extended shelf life further supports the development of annual killifish for use in toxicant assessment.

Further investigations with food (Appendix II) demonstrate large nutritional differences in commercially available brine shrimp. Our comparative study, significantly demonstrates the superiority of enriched San Francisco Brand over another, High Sierra. Fish fed San

Francisco Brand demonstrated greater mean increases in weight and length when compared to fish fed High Sierra (Appendix Tables 2-5). Statistical analysis is presented in Appendix IV.

Preliminary studies on other species of annual killifish that are easier to culture indicate that they too are capable of long term storage and easily hatched in kit form. If annual killifish embryos prove to be good candidates for toxicant assessment, a large choice (over 100 species) exist that may vary in their sensitivity and therefore application. The culture of one other annual fish (e.g. Blue Gularis) was found to be easier, due to the larger size of newly hatched fry and their less selective feeding behavior. Further investigations may be worth while to determine which genus of annual killifish are the easiest to maintain.

CONCLUSIONS

The continuous production of embryos from the annual killifish, *N. guentheri* was accomplished. Techniques developed under this contract permit the economical mass production of these fish and embryos for the first time. Later developed shipment and storage techniques permit these embryos to be delivered in kit form to collaborating researchers in a ready to use fashion, avoiding routine maintenance problems associated with other species. These same embryos may be stored for several months prior to use. Methods described in Phase I and II provide the researcher with the basic background required for lab production of annual killifish embryos for the purpose of toxicant assessment.

Future efforts should center around testing other species of annual killifish to determine benefit of one over another. Additional research that would benefit several areas of science is the identification and isolation of "Diapause Factor", the chemical/protein messenger, that is passed from the adult female, in response to specific environmental stimuili, to the egg, in order to control diapause. This growth suspending factor, if identified, has a variety of potential applications that benefit mankind, such as cellular inhibition for the purpose of treating cancer or premature aging. In addition, findings indicate that embryonic diapause in fish is similar to insect diapause. The same diapause factor may work on insects, permitting the manipulation of their normal development and diapause onset. The present use of chemical toxins such as pesticides could be replaced or reduced with biological compounds targeting insect development. Insect diapause permits their hatching out during favorable conditions which coincides with crop growth. If their developmental cue could be broken, insects could be tricked to prematurely hatch at a time when their survival is impossible.

BIBLIOGRAPHY

Andrewartha, H.G., 1952. Diapause in relation to the ecology of insects. Biol. Rev. Cambridge Phil.Soc., 27: 50.

Bailey, R.G., 1972. Observations on the biology of Nothobranchius guentheri (Pfeffer)(Cyprinodontidae), an annual fish from the coastal region of East Africa. African Journal Hydrobiology and Fisheries, 2: 33-43.

Ballard, W.W., 1973. Normal embryonic stages for salmonid fishes, Based on Salmo gairdneri and Salvelinus fontanalis J.Exp.Zool., 184: 7-26.

Beck, Stanely D., 1968. Insect Photoperiodism. Academic Press, Inc., New York, New York. 265pp.

Boyd, Claude E. and Frobish, Lowell T., 1990. Water Quality in Ponds for Aquaculture. Birmingham Publishing Company, Birmingham, Alabama. 482pp.

Di Michele, Leonard and Taylor, Malcolm H., 1981. "The Mechanism of Hatching in Fundulus heteroclitus: Development of Physiology." J.Exp.Zool., 217: 73-79.

Di Michele, Leonard, Taylor, Malcolm H. and Singleton, Rivers Jr., 1981. "The Hatching Enzyme of Fundulus heteroclitus" J.Exp.Zool., 216: 133-140.

Haas, Richard, 1976a. Behavioral biology of the annual killifish, Nothobranchius guentheri. Copeia, 1: 80-91.

Hass, Richard, 1976b. Sexual selection in Nothobranchius guentheri (Pisces: Cyprinodotidae). Evolution, 30: 614-622.

Hawkins, A.D., 1981. Aquarium Systems. Academic Press Limited/Academic Press, Inc., San Diego, California. 452pp.

Hull, Eugene, 1986. The role of light in controlling the onset and duration of embryonic diapause in Nothobranchius guentheri. Ph.D. Thesis, New York University, 150 pages.

Hull, Eugene, 1993 The use of diapausing eggs from the annual killifish for rapid and sensitive biological screening of toxicants in the aquatic environment. Report # AD-A289056, U.S. Army Medical Research and Development Command, Fort Detrick, MD. 32pp

Inglima, Kenneth, 1980. Reversible stage-specific embryonic inhibition mediated by an adult-produced diffusible substance in the annual fish, Nothobranchius guentheri. New York University, M.S. thesis, 125.

Inglima, Kenneth, Perlmutter, Alfred, and Markofsky, Jules, 1981. Reversible stage-specific embryonic inhibition mediated by the presence of adults in the annual fish Nothobranchius guentheri. Journal of Experimental Zoology, 215: 23-33.

Ingram, Michael Dr., 1985. Clearwater Guide to Controlled Environment Re-Circulating Systems. Clearwater Publishing Limited, Isle of Man, British Isles. 72pp.

Jubb, R.A., 1969. The Nothobranchius(Pisces Cyprinodontidae) of Southern Africa and a new species from Lake Chilwa, Malawi. Annals of the Cape Provincial Museums (Natural History), 8: 1-11.

Klee, J.A., 1965. Quick review of Nothobranchius. J. Amer. Killifish Assn., 2: 11-16.

Lees, A.D., 1955. The Physiology of Diapause in Arthropods, Cambridge University Press, New York, New York. 150.

Levels, P.J., Gubbels, R.E.M.B. and Denunce, J.M., 1986. "Oxygen Consumption During Embryonic Development of the Annual Fish Nothobranchius guentheri with special reference to Diapause." Comp.Biochem.Physiol., Vol. 84A, No.4, pp 767-770.

Markofsky, Jules, and Matais, Jonathan R., 1977. The Effects of temperature and season of collection on the onset and duration of diapause in embryos of the annual fish Nothobranchius guentheri. J.Exp.Zool.,202: 49-56.

Markofsky, Jules, and Matais, Jonathan R., 1978. Effects of light-dark cycles and temperature on embryonic diapause in the East African annual fish, Nothobranchius guentheri. Proc.3rd Int.Conf.Soc. Chronobiology, 269-275.

Markofsky, Jules, and Milstoc, Mayer, 1979. "Histopathological Observations of the Kidney During Aging of the Male Annual Fish Nothobranchius guentheri." Exp.Gernotol., Vol 14, pp149-155.

Markofsky, Jules, Matias, J.R., Inglima, K., Vogelman J.H. and Orentreich, N., 1979. "The Variable Effects of Ambient and Artificial light: Dark cycles on Embryonic Diapause in a Laboratory Population of the Annual Fish Nothobranchius guentheri." Journal of Exp. Biol., Vol. 83: 203-215.

Markofsky, Jules, and Perlmutter, A., 1972. Age at sexual maturity and its relationship to longevity in the male annual cyprinodont fish, Nothobranchius guentheri, Exp.Gerontol., 7: 131-135.

Markofsky, Jules, and Perlmutter, A.,1973. Growth differences in subgroups of varying longevities in a laboratory population of the male annual cyprinodont fish, Nothobranchius guentheri. Exp.Gerontol., 8: 65-73.

Myers, George S., 1942. Studies on South American fresh water fishes. I. Stanford Ichth.Bull., 2: 89-114.

Myers, George S., 1952. Annual fishes. Aquarium Journal, 23: 125-141.

Oppenheimer, J.M., 1937. The Normal stages of Fundulus heteroclitus. Anat. Rec., 68: 1-15.

Oppenheimer, J.M., 1947. Organization of the teleost blastoderm. Quart.Rev. Biol.,22: 105-118.

Peters, N., 1963. Embryonale anpassungen oviparer zahnkarpfen aus periodisch austroknenden gewasseum, Int.Rev.Ges. Hydrobiol., 18: 257-313.

Rand, Gary M. and Petrocelli, Sam R., 1985. Fundamentals of Aquatic Toxicology. Hemisphere Publishing Corporation, New York, New York. 666pp.

Simpson, Betty R.C., 1979. The phenology of annual killifishes. Symp.Zool.Soc.Lond.,44: 243-261.

Spotte, Stephen, 1979. Fish and Invertebrate Culture. Wiley-Interscience Publication, New York, New York. 179 pp.

Trikaus, J.P., 1951. A study of the mechanism of epiboly in the egg of Fundulus heteroclitus.J.Exp.Zool.,118: 269-319.

Trikaus, J.P., 1963. The cellular basis of Fundulus Epiboly, adhesivisity of blastula and gastrula cells in culture. Develop.Biol.,7: 513-532.

Turner, B.J., 1964. An introduction to the fishes of the genus Nothobranchius. Afr.Wildlife,18: 117-124.

Wourms, John Barton, 1964. Comparative observations of the early embryology of Nothobranchius taeniopygus (Hilgendorf) and Aplocheilicthyspumilis (Boulenger) with special reference to the problem of naturally occurring embryonic diapause in teleost fishes. Ann. Report East African Fresh Water Fisher.Instr.,App. H., 68-73.

Wourms, John Barton, 1966. A naturally occurring vertebrate dispersion-reaggregation system subject to developmental arrest. Stanford University PhD.Thesis, 1-155.

Wourms, John Barton, 1967. Annual fishes In: Methods in Developmental Biology, Wilt Fred H., and Wessells, Norman K.(eds.), Thomas Y. Crowell Company, New York, New York, 123-137.

Wourms, John Barton, 1972a. Developmental biology of annual fishes: I. Stages in the normal development of Austrofundulus myersi Dahl. Journal of Experimental Zoology, 182: 143-168.

Wourms, John Barton, 1972b. The developmental biology of annual fishes: II. Naturally occurring dispersion and reaggregation of blastomeres during the development of annual fish eggs. Journal of Experimental Zoology,182: 169-200.

Wourms, John Barton, 1972c. The developmental biology of annual fishes: III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. Journal of Experimental Zoology, 182: 389-414.

APPENDIX I

Heat production by a battery operated package heater.

When shipping eggs to collaborating USABRDL during winter months, viability was reduced due to freezing temperatures. Previous studies demonstrated temperature fluctuation as the cause for hatching. To avoid rapid change in temperatures during shipping, eggs were packed in foam containers and heat packs placed on the outside. This method of heat propagation was poor as commercially purchased heat packs (Grabber International) would lose their potency if not shaken. To provide a better heater for winter shipment a battery operated heater was developed by using a battery and resistor which essentially shorts circuits the battery over time while emitting heat.

This package heater is based on a resistive heater element and a battery. The battery is inserted into the heater upon shipping the container and will create heat based on the battery's discharge characteristics. The amount of heat generated is small and to keep the internal compartment above ambient temperature depends on a sufficient amount of thermal insulation in the package.

The battery is 1.5 volt alkaline "C" cell and the heater element is a 1/2 watt 4.7 ohm resistor. The battery voltage begins at 1.5 volts and drops to .75 volts by the end of its life. To get the power generated at the beginning and end of the battery life we use the following formulas:

P = power in watts I = current in amps V = voltage in volts

R = resistance in ohms

I = V / R	I = 1.5 / 4.7	.319 amps at start
	I = 0.75 / 4.7	.160 amps at end
P = I x V	P = .319 x 1.5	.479 watts at start
	P = .160 x .75	.12 watts at end

Heat flows from warm to cold. In this heater the resistor is the hottest element. It warms the surrounding air in the internal compartment. It may be possible to provide a good thermal conduction path from the resistor to the egg container which would then make the egg temperature greater than the internal compartment air. However, since there is no temperature control it may be possible to raise the egg temperature too high if we do this. The heated internal compartment air transfers heat through the Styrofoam and plastic to the outside air which will be referred to as the ambient temperature. If there is no direct conduction path from the resistor to the eggs then the eggs will be near the temperature of

the air in the internal compartment. This is the temperature that we are concerned with and the temperature that will be monitored. The temperature inside the compartment will be a function of the wattage dissipated by the heater and the thermal resistance of the container. Thermal resistance is determined by the physical properties of the materials. The amount of heat flow is determined by the thermal resistance and the surface area available for the heat to flow through. Since the Styrofoam container has a fixed surface area we will be concerned with the thermal resistance as a complete system from the internal compartment air to the outside air. The thermal resistance (TR) will be expressed as the temperature rise above ambient per watt of power dissipated inside the chamber.

T = temp. inside chamber TA = ambient temp.

$TR = (T - TA) / P$

The experimentally measured thermal resistance for the Styrofoam wrapped in plastic inside the cardboard box was approximately 150° F per watt. Using this value with the starting and power dissipation the temperature rise above ambient would be as follows:

T rise = .479 x 150 = 72° F at start

T rise = .12 x 150 = 18° F at end

If the outside air was 20° F the temperature in the compartment would be 92° F at start and 38° F at the end. If the ambient temperature was 60° F when installing the battery the temperature in the chamber would be 132° F. (This may require refrigerating the battery before installation to flatten out the beginning temperature curve.) According to the data available from Duracell, the battery should end its performance at about 30 hours. This endurance may be reduced however if the battery temperature is low. This may be a good reason to place the resistor close to or touching the battery in order to get the maximum performance from the battery. The battery life can also be extended by using a larger resistor which will reduce the current drain and lower the power dissipation. This however will lower the temperature rise of the internal compartment.

Additional duration can be obtained by controlling the temperature or adding additional batteries. One of the drawbacks of the simple resistor heater is that it generates the greatest temperature rise at the start of the cycle when it is not needed and generates the least temperature at the end of the cycle when it may be needed the most. The temperature rise can be increased by increasing the thermal resistance of the container. Heat generation is reported in Appendix **Table 1** below.

Results

A simple battery heater is capable of maintaining internal temperature during shipment in the coldest months to avoid mortality associated with extreme drops in temperature. The cost of production of such unit is less than $2.00/unit making it both a viable and economic method for shipping.

Appendix Table 1

The ability of battery operated package heater to generate heat. A package heater was activated and placed inside a 2" foam container. Temperature readings were generated from a thermal probe placed inside the compartment. The foam container was placed in a commercial freezer immediately after the battery was activated.

		Radio Shack "C" alkaline cell	
Time	Deg F	Battery V	Time plot
20:30	75.5	1.45	20.5
20:35	76.9	1.45	20.58333
20:45	76.1	1.43	20.75
21:00	73.6	1.41	21
21:15	70.7	1.37	21.25
21:30	69.2	1.35	21.5
21:45	66.6	1.32	21.75
22:05	64.4	1.3	22.08333
22:40	61.2	1.25	22.66667
23:00	59.8	1.25	23
12:00	55.7	1.2	24
01:00	52.7	1.15	25
02:00	50.8	1.125	26
03:45	48	1.075	27.75
07:00	46.1	1	31
08:00	44.6	0.975	32
00:00	40.5	0.925	36
13:00	39.5	0.85	38
14:00	38.4	0.775	40
16:40	21.8	0.125	42.6
17:15	19.2	0.1	43.25
21:30	12	0.065	47.5

APPENDIX II

The effects of vitamin/amino acid enriched brine shrimp on growth. Brine shrimp that was enriched with a combination of yeast and spirulina was compared to brine shrimp alone.

During routine care of these fish it was observed that various commercially available foods, especially the staple diet frozen brine shrimp, would be rejected by some fish or increase mortality in fish. To study this phenomenon, we compared a nutritionally supplemented frozen brine shrimp available from San Francisco Brand Name Brine Shrimp, to High Sierra brand brine shrimp available from pet stores. The purpose of this study was aimed at identifying safe foods for the growth and maintenance of these fish, that also provide the maximum nutrition.

A modified flow through system was used in testing the annual killifish. The fish were housed in 9 liter V-shaped tanks with a bottom drain pipe covered by nylon mesh. Each tank contained one air tube and one PVC grid for hiding. Carbon filtered well water was adjusted to 1 gram per liter with NaCl, and buffered to a final pH of 6.9-7.1 (see water conditioning, Phase 1 Report). Water was stored in all glass 200 gallon tanks raised to a height above the fish. Water flowed by gravity through PVC pipes fitted with clamp valves to control flow to each tank. Valves were adjusted to permit a slow change of 100-125% of their water daily. Lighting was accomplished by 4 GE 48" cool white fluorescent bulbs. The Light Dark cycle was 13:11 at an intensity of 100-115 ft. candles

.

Fish were randomly selected on the same day after a 24 hour starvation period from 50 day old laboratory stock of annual killifish, *N. guentheri*. One hundred females ranging in length from 1.5cm to 2.8 cm and wet weight of 0.10 to 0.50 g were randomly placed 5 fish per tank in each of 20 tanks. Ten tanks were randomly selected for each food treatment. One group received HighSierra Brand frozen adult brine shrimp, the other received supplemented adult frozen brine shrimp available from San Francisco Bay Brand. Fish were fed in excess 3 times daily at 9:00 am, 1:00pm, and 4:00pm. Any visible food that did not exit via the drain pipe was removed 15-20 minutes after feeding. After 36 days, the ending weights and lengths were measured for comparison to beginning values. Data are presented below as mean beginning lengths, ending lengths, beginning weight, ending weight, mean increase in length and weight and combined mean increase in length and weight.

In general growth as measured by increase in length and weight was slow when compared to similar fish routinely grown in our lab that receive a variety diet. However, there was an obvious benefit observed in growth in fish fed San Francisco brand brine shrimp vrs. High Sierra. San Francisco brand led to mean increase in length and weight of 0.56cms and 0.16 grams respectively . High Sierra led to a smaller mean increase in length and weight of 0.13 cm and -0.02 grams (Appendix Tables 2-5). Fish fed High Sierra brand actually lost weight and therefore this food has little or no nutritional value at all.

Appendix Table 2

Mean lengths and weights of 50 day old *Nothobranchius* fed San Francisco Bay Brand Brine Shrimp™ for 36 days.

Mean lengths & weights of 50 day old *Nothobranchius* fed San Francisco Bay Brand Brine Shrimp™ for 36 days

Replicate Number	Beg. Lengths	Mean Beg. Lengths	Ending Lengths	Mean Ending Lenths	Beg. Weights	Mean Beg. Weights	Ending Weights	Mean Ending Weights
1	1.89 cm		2.45 cm		.11 g		.45g	
	2.00 cm		2.55 cm		.19 g		.35 g	
	1.86 cm		2.25 cm		.21 g		.30 g	
	1.80 cm		2.58 cm		.19 g		.35 g	
	1.50 cm	1.81 cm	2.60 cm	2.49 cm	.10 g	.16 g	.50 g	.39 g
2	1.70 cm		2.00 cm		.20 g		.20 g	
	1.70 cm		2.10 cm		.13 g		.25 g	
	1.84 cm		2.17 cm		.17 g		.25 g	
	1.70 cm		2.30 cm		.12 g		.33 g	
	2.20 cm	1.83 cm	*	2.14 cm	.40 g	.20 g	*	.26 g
3	1.79 cm		2.90 cm		.18 g		.40 g	
	2.10 cm		1.90 cm		.20 g		.20 g	
	1.75 cm		2.31 cm		.11 g		.30 g	
	1.80 cm		2.60 cm		.17 g		.32 g	
	2.50 cm	1.99 cm	2.65 cm	2.47 cm	.50 g	.23 g	.50 g	.34 g
4	1.80 cm		2.50 cm		.19 g		.44 g	
	1.80 cm		2.20 cm		.15 g		.31 g	
	1.58 cm		2.30 cm		.16 g		.30 g	
	1.70 cm		2.49 cm		.12 g		.35 g	
	1.70 cm	1.72 cm	2.35 cm	2.37 cm	.21 g	.17 g	.31 g	.34 g
5	1.52 cm		2.40 cm		.12 g		.35 g	
	1.93 cm		2.50 cm		.20 g		.39 g	
	1.88 cm		2.50 cm		.29 g		.44 g	
	1.85 cm		2.60 cm		.15 g		.41 g	
	1.60 cm	1.76 cm	2.20 cm	2.44 cm	.25 g	.20 g	.25 g	.37 g
6	1.79 cm		2.50 cm		.19 g		.40 g	
	1.80 cm		2.40 cm		.20 g		.35 g	
	1.80 cm		2.40 cm		.16 g		.35 g	
	2.00 cm		2.50 cm		.45 g		.45 g	
	2.00 cm	1.88 cm	2.30 cm	2.42 cm	.30 g	.26 g	.34 g	.38 g
7	1.90 cm		2.50 cm		.12 g		.55 g	
	2.00 cm		2.60 cm		.21 g		.50 g	
	1.68 cm		2.20 cm		.13 g		.33 g	
	1.75 cm		1.80 cm		.30 g		.16 g	
	2.20 cm	1.91 cm	2.65 cm	2.35 cm	.45 g	.24 g	.45 g	.40 g
8	1.69 cm		2.40 cm		.11 g		.35 g	
	1.87 cm		2.60 cm		.15 g		.55 g	
	1.83 cm		2.60 cm		.18 g		.45 g	
	2.00 cm		2.50 cm		.20 g		.45 g	
	2.30 cm	1.94 cm	*	2.53 cm	.60 g	.25 g	*	.45 g
9	1.72 cm		2.05 cm		.14 g		.35 g	
	1.79 cm		2.60 cm		.16 g		.55 g	
	1.80 cm		2.60 cm		.16 g		.45 g	
	1.70 cm		2.50 cm		.20 g		.45 g	
	2.20 cm	1.84 cm	*	2.44 cm	.30 g	.19 g	*	.45 g
10	1.76 cm		2.20 cm		.19 g		.33 g	
	1.75 cm		2.00 cm		.20 g		.24 g	
	1.70 cm		2.40 cm		.14 g		.38 g	
	1.60 cm		2.40 cm		.10 g		.35 g	
	2.00 cm	1.76 cm	2.60 cm	2.32 cm	.50 g	.23 g	.41 g	.34 g
TOTALS	92.12 cm	1.84 cm	112.70 cm	2.40 cm	10.66 g	.21 g	17.44 g	.37 g

Appendix Table 3

Mean lengths and weights of 50 day old *Nothobranchius* fed High Sierra Brand™ Brine shrimp for 36 days.

Mean lengths & weights of 50 day old *Nothobranchius* fed High Sierra Brand Brine Shrimp™ for 36 days.

Replicate Number	Beg. Lengths	Mean Beg. Lengths	Ending Lengths	Mean Ending Lengths	Beg. Weights	Mean Beg. Weights	Ending Weights	Mean Ending Weights
1	1.71 cm		1.95 cm		.14 g		.25 g	
	1.69 cm		2.10 cm		.12 g		.30 g	
	1.70 cm		1.83 cm		.15 g		.15 g	
	1.80 cm		1.70 cm		.13 g		.14 g	
	2.80 cm	1.94 cm	2.10 cm	1.94 cm	.30 g	.17 g	.18 g	.20 g
2	1.70 cm		2.05 cm		.15 g		.19 g	
	1.80 cm		1.95 cm		.16 g		.20 g	
	1.55 cm		1.78 cm		.19 g		.10 g	
	1.85 cm		1.65 cm		.15 g		.10 g	
	1.50 cm	1.68 cm	1.90 cm	1.87 cm	.18 g	.17 g	.18 g	.15 g
3	1.70 cm		1.80 cm		.16 g		.13 g	
	1.65 cm		1.70 cm		.16 g		.10 g	
	2.10 cm		2.10 cm		.28 g		.15 g	
	1.50 cm		1.90 cm		.11 g		.15 g	
	2.00 cm	1.79 cm	2.30 cm	1.96 cm	.30 g	.20 g	.24 g	.15 g
4	1.88 cm		2.00 cm		.17 g		.21 g	
	1.80 cm		2.40 cm		.16 g		.30 g	
	1.70 cm		2.10 cm		.12 g		.15 g	
	1.70 cm		1.90 cm		.18 g		.17 g	
	1.80 cm	1.78 cm	1.75 cm	2.03 cm	.50 g	.23 g	.09 g	.18 g
5	1.65 cm		1.80 cm		.15 g		.11 g	
	2.10 cm		1.70 cm		.21 g		.10 g	
	1.70 cm		2.20 cm		.13 g		.27 g	
	1.90 cm		1.70 cm		.19 g		.11 g	
	1.70 cm	1.81 cm	1.80 cm	1.84 cm	.30 g	.20 g	.13 g	.14 g
6	1.69 cm		2.00 cm		.11 g		.16 g	
	1.81 cm		1.70 cm		.12 g		.10 g	
	1.61 cm		2.15 cm		.14 g		.21 g	
	1.95 cm		2.00 cm		.20 g		.14 g	
	1.70 cm	1.75 cm	*	1.96 cm	.10 g	.13 g	*	.15 g
7	1.89 cm		1.70 cm		.22 g		.10 g	
	1.52 cm		1.80 cm		.08 g		.10 g	
	2.05 cm		1.95 cm		.21 g		.15 g	
	1.60 cm		2.00 cm		.17 g		.19 g	
	1.70 cm	1.75 cm	*	1.86 cm	.14 g	.16 g	*	.14 g
8	1.79 cm		1.70 cm		.11 g		.13 g	
	2.05 cm		1.70 cm		.27 g		.20 g	
	2.05 cm		2.10 cm		.28 g		.20 g	
	1.80 cm		1.80 cm		.18 g		.10 g	
	1.55 cm	1.85 cm	2.00 cm	1.86 cm	.11 g	.19 g	.19 g	.16 g
9	1.75 cm		1.90 cm		.17 g		.15 g	
	1.65 cm		2.10 cm		.15 g		.20 g	
	1.70 cm		2.00 cm		.17 g		.15 g	
	1.70 cm		2.10 cm		.19 g		.18 g	
	1.80 cm	1.72 cm	1.80 cm	1.98 cm	.20 g	.18 g	.10 g	.16 g
10	1.68 cm		1.70 cm		.37 g		.09 g	
	1.76 cm		1.70 cm		.19 g		.10 g	
	1.65 cm		1.90 cm		.18 g		.11 g	
	1.80 cm		1.80 cm		.16 g		.10 g	
	1.75 cm	1.73 cm	2.10 cm	1.84 cm	.13 g	.21 g	.20 g	.12 g
TOTALS	88.98 cm	1.78 cm	91.86 cm	1.91 cm	9.14 g	.18 g	7.55 g	.16 g

Appendix Table 4

Mean increase of length and weight in 50 day old *Nothobranchius* fed San Francisco Brand Brine Shrimp™ for 36 days.

Mean increase of length & weight in 50 day old *Nothobranchius* fed San Francisco Brand Brine Shrimp™ for 36 days.

FOOD BRAND	TANK NUMBER	MEAN INCREASE IN LENGTH (cm)	MEAN INCREASE IN WEIGHT (g)
San Francisco Bay	1	0.68	0.23
San Francisco Bay	3	0.31	0.06
San Francisco Bay	5	0.48	0.11
San Francisco Bay	6	0.65	0.17
San Francisco Bay	7	0.68	0.17
San Francisco Bay	10	0.54	0.12
San Francisco Bay	12	0.44	0.16
San Francisco Bay	15	0.59	0.2
San Francisco Bay	18	0.60	0.26
San Francisco Bay	20	0.56	0.12
COMBINED/ TOTALS		0.56	0.16

Appendix Table 5

Mean increase of length and weight in 50 day old *Nothobranchius* fed High Sierra Brine Shrimp™ for 36 days.

Mean increase of length & weight in 50 day old Nothobranchius fed High Sierra Brine Shrimp™ for 36 days.

FOOD BRAND	TANK NUMBER	MEAN INCREASE IN LENGTH (cm)	MEAN INCREASE IN WEIGHT (g)
High Sierra	2	0.00	0.03
High Sierra	4	0.20	-0.02
High Sierra	8	0.17	-0.05
High Sierra	9	0.25	-0.04
High Sierra	11	0.03	-0.06
High Sierra	13	0.21	0.02
High Sierra	14	0.11	-0.02
High Sierra	16	0.01	-0.03
High Sierra	17	0.26	-0.02
High Sierra	19	0.11	-0.09
COMBINED/ TOTALS		0.13	-0.02

APPENDIX III

General methodology utilized for preparation and shipment of embryos used for toxicant assessment at collaborating USABRDL.

Eggs were harvested from the general breeding population of killifish housed in a recirculating aquaculture system. Embryos aged 1-7 days were harvested by stirring the sand bottom and swiftly moving a net in a figure eight configuration. The eggs are somewhat buoyant and float in the water for several seconds after stirring the sand, making their removal simple. Tanks were immediately filtered with diatomaceous earth and carbon to remove bacteria and fungus released into the water from the sand substrate. Eggs were immediately placed in freshly prepared stock water of an identical temperature. Eggs were rinsed several times and placed in large shallow glass trays at a density of not more than 1 egg per 2 mls stock water. Eggs were rinsed daily and stored in the dark at a temperature of approximately 25.0°C. When embryos develop to stages 24-30, viable embryos were selected and placed in a plastic bag containing sterile Canadian peat moss. The peat moss was first boiled several times, processed to pass through a 0.6 mm mesh screen and squeezed until damp to the touch. The peat moss containing eggs were wrapped with a minimum of 1 inch of foam and transported by next day air to collaborating researchers. A package heater or commercially purchased heat pack was used to maintain temperature in the coldest winter months.

When received by collaborating researchers the eggs are incubated for 30 days or longer depending when required for any study. Eggs are easily harvested by passing the peat moss through a 0.6mm mesh which lets the soil pass while capturing the embryos. The eggs are hatched by crowding in a vial. A density of 20 eggs per ml is used to rapidly initiate the hatching process.

Eggs can be manipulated prior to insertion into peat moss for long term storage. These embryos would be harvested from fish exposed to short day (L:D, 10:14) conditions for 100 or more days and incubated at 21.0°C. Embryos would be shipped to collaborating researchers and stored at 21.0°C. When embryos are required for toxicant assessment, they are maintained at room temperature of 24.0-28.0°C for 60 days permitting the embryos to bypass Diapause II and develop to Diapause III the prehatching stage. When fully developed they are collected and handled as stated previously. Once in Diapause III, eggs have a shelf life of 90 days.

APPENDIX IV

Part 1. Statistical analysis for experimental designs A - G.

Part 2. Statistical analysis for growth study (Appendix II).

APPENDIX IV

Dr. Raid W. Amin
8585 Belle Meadow Blvd.
Pensacola, FL 32514
(904) 477-7283 (H)
(904) 474-3014 (W)

STATISTICAL CONSULTING REPORT
FOR Dr. EUGENE HULL

Part I: Hatching Ratios of Eggs.

Description of the statistical test performed: The results of each 4 replications were pooled together for each day. The 2x2 tables were analyzed for each day utilizing both an exact nonparametric permutation test and an asymptotic χ^2 test with the software StatXact-Turbo. It should be noted that the the results from Day=0 to Day=20 are not independent, and no comparisons should be made between the different days. If the pre-conceived alternative hypothesis is one-sided, then the p-values for the one-sided tests should be used. The resulting p-values are as follows.

A. The Effect of Drops in Temperature on Hatching and Viability in Water Incubated Eggs.

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	**0.0403**	0.0807	0.0400	0.0801
8	0.0233	0.0465	0.0230	0.0461
12	0.0074	0.0149	0.0073	0.0147
20	0.0029	0.0059	0.0029	0.0058

B. The Effect of Drops in Temperature on Hatching and Viability in Water Incubated Eggs that were Dried for Ten Days Prior to Being Returned to Water Incubation.

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	**0.0014**	0.0029	0.0014	0.0028
8	0.0001	0.0002	0.0001	0.0002
12	0.0000	0.0000	0.0000	0.0000
20	0.0000	0.0000	0.0000	0.0000

C. The Effect of Drops in Temperature on Hatching and Viability in Dried Eggs that are Stored on Moist Filter Paper.

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	**0.0278**	0.0556	0.0276	0.0551
8	0.0028	0.0056	0.0027	0.0055
12	0.0017	0.0034	0.0017	0.0034
20	0.0009	0.0018	0.0009	0.0017

D. The Effect of Drops in Temperature on Hatching and Viability in Water Incubated Eggs that were Programmed to Enter DII for 20 Days.

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	**0.0046**	0.0091	0.0045	0.0090
8	0.0002	0.0003	0.0002	0.0003
12	0.0000	0.0000	0.0000	0.0000
20	0.0000	0.0000	0.0000	0.0000

E. The Effect of Drops in Temperature on Hatching and Viability in Dried Eggs that are Developed to Stage 38-40 Prior to the Pre-hatching Stage.

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	0.9999	0.9999	0.9999	0.9999
8	0.9999	0.9999	0.9999	0.9999
12	0.1597	0.3194	0.1592	0.3183
20	0.3423	0.6847	0.3420	0.6840

F. The Effect of Reducing Egg Water Content on Hatching and Viability in Embryos Exposed to a $4C^0$ Drop in Temperature (3g/L vs 10g/L).

	Permutation Test		χ^2-Test	
Day	1-sided	2-sided	1-sided	2-sided
0	0.9999	0.9999	0.9999	0.9999
4	0.9999	0.9999	0.9999	0.9999
8	0.1587	0.3173	0.1581	0.3163
12	**0.0437**	0.0874	0.0433	0.0867
20	0.0216	0.0432	0.0214	0.0427

G. The Effect of Short day Adult Exposure on Long Term Storage of Embryos Dried in Peat Moss and Stored at $21C^0$.

The four replicates for each Day (90, 180, 270, 300) were pooled together. The sample survival rates of embryos developed greater than DII is denoted by $\hat{P}$, and the survival rate of embryos developed less than or equal to DII is denoted by $1-\hat{P}$. The table below presents the point estimates and 95% confidence intervals for the parameter P at each stage (Day=90, 180, 270, 300).

Day	Point Estimate	Confidence Interval
90	$\hat{p}=0.02609$	$0.0054 \leq P \leq 0.0744$
180	$\hat{p}=0.2056$	$0.1336 \leq P \leq 0.2946$
270	$\hat{p}=0.5610$	$0.4470 \leq P \leq 0.6705$
300	$\hat{p}=0.7692$	$0.6481 \leq P \leq 0.8647$

Part II: Change in Length and Weight in 50 Day Old Nothobranchius Fed Two Types of Shrimp : (High Sierra vs San Francisco Bay)

Statistical Analysis: A Repeated Measures Design was utilized to analyze the data, separately for variables length and weight.

Model : Response = Subject(Group) Group Time Group*Time

Note: Subject(Group) denote (Subjects are nested in Groups). All data points were used, except when there were missing values. The Statistical Analysis System (SAS) was utilized, using the GLM procedure for repeated measures designs. The enclosed SAS printouts show detailed work and results. The first ten pages of the SAS printout give summary statistics and box plots for each group.

1. Variable=Length: (SAS printout pages 12, 14, 15). The interaction effect Group*Time is significant (p=0.0001), and this implies that we should not consider the main effect Group averaged over Time. The interaction plot and the accompanying Tukey's multiple comparison procedure led to the following conclusions (at α=0.05):

1. There is a significant increase in length (beginning vs ending) for both groups.

2. Even though both groups had similar beginning lengths, the group that was fed San Francisco Bay shrimp had at the end a significantly higher length than the other group (fed High Sierra shrimp).

2. Variable=Weight: (SAS printout pages 13, 14, 16). The interaction effect Group*Time is significant (p=0.0001), and this implies that we should not consider the main effect Group averaged over Time. The interaction plot and the accompanying Tukey's multiple comparison procedure led to the following conclusions (at α=0.05):

1. There is no significant change in weight for the first group (fed High Sierra shrimp).

2. There is a significant increase in weight for the second group (fed San Francisco Bay shrimp) .

Overall Conclusions:

1. The group that was fed High Sierra shrimp increased in length but did not increase in weight.

2. The group that was fed San Francisco Bay shrimp increased in length and in weight.

3. The increase in length is more dramatic for the group that was fed San Francisco Bay shrimp.